Reaction of Reduced Disulfide Bonds in α-Lactalbumin and β-Lactoglobulin with Acrylonitrile

LEOPOLD WEIL AND THOMAS S. SEIBLES

Reduction of disulfide bonds of α-lactalbumin and β-lactoglobulin with β-mercaptoethanol and subsequent reaction with acrylonitrile was shown to be specific and was confined to the thiol groups, as determined by total amino acid analysis of the protein derivatives. The reaction results in the quantitative and specific conversion of the cysteinyl residues to *S*-cyanoethylcysteinyl groups. Upon acid hydrolysis, the *S*-cyanoethylcysteinyl groups were converted quantitatively to *S*-carboxyethylcysteine and were determined analytically as such.

INTRODUCTION

Cleavage of disulfide bonds in proteins is of primary importance in elucidating the structure of proteins. Methods used in the past (1–4) although useful in certain cases, have not always proved to be applicable as general procedures.

A few years ago Tomimatsu, Bartulovich, and Ward (5) in a preliminary note proposed the possible use of acrylonitrile for the conversion of the sulfhydryl groups in wool keratin derivatives. The reaction was based on the conversion of the thiol groups by addition of acrylonitrile to their cyanoethyl derivatives. Since no analytical data were presented by these authors concerning the specificity and quantitative aspects of this reaction, investigations were undertaken to study these phases as applied to α-lactalbumin and β-lactoglobulin.

EXPERIMENTAL

Material

α-Lactalbumin was prepared from pooled milk according to the procedure of Gordon and Ziegler (6) and was crystallized three times. β-Lactoglobulin was obtained as a by-product during the isolation of α-lactalbumin and was crystallized four times.

[1] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

Preparation of *S*-Cyanoethyl-α-lactalbumin and *S*-Cyanoethyl-β-lactoglobulin

One gram of protein, dissolved in water by adjusting the pH to 8.0 with alkali, was reduced with 1.56 g. β-mercaptoethanol in a total volume of 20 ml. The reaction mixture was left overnight under nitrogen, after which period 2.12 g. acrylonitrile was added (molar ratio of β-mercaptoethanol to acrylonitrile, 1:2) and then left at room temperature for an additional 4 hr. The reaction was complete after this time, as indicated by the disappearance of the characteristic odor of β-mercaptoethanol. The α-lactalbumin was then precipitated with 1 *N* HCl at pH 4.0, while in the case of the β-lactoglobulin derivative, the precipitation was carried out at pH 5.0. The precipitated proteins were dialyzed against distilled water at 4° and freeze-dried. The recovery was quantitative. The specific optical rotation was (α^{25}_{D}) of *S*-cyanoethyl-α-lactalbumin dissolved in water at pH 7.5 was −79.5°. The native protein under identical conditions gave a rotation of −59.0°. No measurement for the β-lactoglobulin derivative was carried out since at pH 8.0 (used for the reaction) the protein alone undergoes denaturation and increase in rotation. Both protein derivatives gave clear water solutions at pH 7 and above.

Preparation of *S*-Cyanoethylcysteine

Ten grams of cysteine. $HCl.H_2O$ was dissolved in water, and, after adjusting the pH to 8.0 with 1 *N* NH_4OH, the solution was made up to a volume of 50 ml. After addition of 6 g. acrylonitrile (molar ratio of cysteine to acrylonitrile, 1:2), the reaction mixture under nitrogen was left at room temperature for 4 hr. After evaporation to dryness in vacuum, the residue was dissolved in hot ethanol (75% by vol.) and cooled, and crystalline precipitate was filtered and dried. Yield: 82%; m.p. 170.6–172.0° (uncorr.); α_D^{25} +24.0° (in water).

Anal. Calcd. for $C_6H_{10}N_2SO_2$: C, 41.33; H, 5.85; N, 16.13; S, 18.37%. Found: C, 41.26; H, 5.65; N, 16.24; S, 18.21%.

Preparation of *S*-Carboxyethylcysteine

Three grams of cysteine.$HCl.H_2O$ and 3 g. β-bromopropionic acid were dissolved in 30 ml. water and placed in a closed container which was provided with a glass electrode and a buret through which 2 *N* NH_4OH was delivered to maintain the pH at 8.0. The entire operation was carried out under a nitrogen atmosphere at 50°. After about 2.5 hr., no further acid shift in the pH could be observed. The reaction mixture was then adjusted to pH 3.0 with HCl and evaporated to dryness in vacuum. The residue was extracted with ether to remove any unreacted β-bromopropionic acid. The extracted residue was dissolved in hot ethanol (70% by vol.), filtered, and cooled, and the crystalline precipitate formed was filtered and dried. Yield: 56%; m.p. 181.6–183.2° (uncorr.).

Anal. Calcd. for $C_6H_{11}NSO_4$: C, 37.26; H, 5.69; N, 7.24; S, 16.56%. Found: C, 37.16; H, 5.54; N, 7.20; S, 16.57%.

Amino Acid Composition of the *S*-Cyanoethyl Derivatives of α-Lactalbumin and β-Lactoglobulin

Both protein derivatives and their parent proteins were hydrolyzed with 6 *N* HCl (protein to acid ratio, 1:200) in sealed tubes for 24 hr. at 115°. After drying the hydrolyzates in vacuum over P_2O_5, they were taken up in water and aliquots were analyzed by the chromatographic procedure of Moore, Spackman, and Stein (7). The results obtained and presented in Table I show that the reaction between acrylonitrile and the reduced α-lactalbumin and β-lactoglobulin, respectively, was confined specifically and quantitatively to the conversion of cysteine within the protein chain to a new amino acid derivative, which emerged during the chromatography as a distinct peak between the serine and glutamic acid peaks, while the cystine peak was completely eliminated. The rest of the amino acids, as compared with the analysis of the parent protein, were not affected. As a whole, the amino acid analysis after 24 hr. of hydrolysis, as shown in Table I, agreed very well with those reported previously in the literature (8, 9, 11). The identity of the new peak with *S*-carboxyethylcysteine, when the hydrolyzates of the *S*-cyanoethyl derivatives of the proteins were analyzed, was established by spiking the respective hydrolyzates with known amounts of synthetic *S*-carboxyethylcysteine. The addition of this compound to the hydrolyzate was additive to the new peak and the recovery was quantitative. (Calculation was based on the determined color yield of 0.73 for *S*-carboxyethylcysteine.)

It should be mentioned that if the above experi-

TABLE I

Amino Acid Composition after 24 Hr. of Acid Hydrolysis

Grams/100 g. protein

Constituent	α-Lactalbumin	*S*-Cyanoethyl-α-lactalbumin	β-Lactoglobulin	*S*-Cyanoethyl-β-lactoglobulin
Aspartic acid	18.46	18.64	11.86	11.72
Threonine	5.52	5.65	4.98	5.02
Serine	4.43	4.23	3.42	3.30
S-Carboxyethylcysteine	0.00	10.36 (10.29)[a]	0.00	5.56 (5.46)[a]
Glutamic acid	12.70	12.81	19.22	19.40
Proline	1.52	1.40	5.18	5.30
Glycine	3.22	3.20	1.42	1.46
Alanine	2.10	2.02	7.26	7.35
Cystine	4.80 (6.40)[b]	0.00	2.80 (3.40)[b]	0.00
Valine	4.25	4.63	5.75	5.70
Methionine	0.89	0.91	3.28	3.18
Isoleucine	6.48	6.91	6.10	5.98
Leucine	11.38	11.50	15.68	15.47
Tyrosine	5.23	5.14	3.80	3.80
Phenylalanine	4.54	4.61	3.68	3.50
Lysine	11.42	11.50	11.64	11.46
Histidine	2.76	2.60	1.61	1.56
Arginine	1.11	1.23	2.80	2.79
Tryptophan	6.78[c]	6.70[c]	2.70[c]	2.75[c]
Ammonia	1.79	2.64	1.47	1.97
ΔAmmonia	—	0.85	—	0.50

[a] Calculated values based on cystine content reported in literature (8, 9).

[b] Cystine values as reported in literature (8, 9) after oxidation to cysteic acid.

[c] Determined by the method of Spies and Chambers (10).

ments were carried out with an acid-hydrolyzed synthetic sample of *S*-cyanoethylcysteine (hydrolytic conditions were identical with those used for the proteins), subsequent chromatography produced the same result as above.

Similar spiking experiments with synthetic *S*-cyanoethylcysteine resulted only in an increase in a distorted glutamic acid peak while the new peak, which was due to *S*-carboxyethylcysteine (as shown above), was not affected.

It would appear that the *S*-cyanoethylcysteine which was formed from the cysteine residues within the protein chain during the reaction between the acrylonitrile and the reduced proteins was converted to *S*-carboxyethylcysteine during the hydrolysis preparatory to amino acid analysis.

The over-all reactions which took place during the process are visualized in the equations below:

$$\underset{\text{cysteine}}{\text{HOOC—CH(NH}_2\text{)—CH}_2\text{—SH}} + \underset{\text{acrylonitrile}}{\text{CH}_2\text{=CH—C}\equiv\text{N}} \rightarrow \underset{\text{S-cyanoethylcysteine}}{\text{HOOC—CH(NH}_2\text{)—CH}_2\text{—S—CH}_2\text{—CH}_2\text{—C}\equiv\text{N}} \quad (1)$$

$$\text{HOOC—CH(NH}_2\text{)—CH}_2\text{—S—CH}_2\text{—CH}_2\text{—C}\equiv\text{N} \xrightarrow[\text{H}_2\text{O}]{\text{HCl}} \underset{\text{S-carboxyethylcysteine}}{\text{HOOC—CH(NH}_2\text{)—CH}_2\text{—S—CH}_2\text{—CH}_2\text{—COOH}} + \text{NH}_4\text{Cl} \quad (2)$$

According to Eq. (2), hydrolysis of one mole of *S*-cyanoethylcysteine should yield one mole of *S*-carboxyethylcysteine and one mole of ammonia. This was confirmed experimentally inasmch as an acid hydrolyzate of a synthetic sample of *S*-cyanoethylcysteine (hydrolysis incidental with that used for the proteins), upon chromatography, resulted in a stoichiometric formation of *S*-carboxyethylcysteine and ammonia. A similar conclusion can be reached if one compares the amounts of ammonia (see Table I) produced during the acid hydrolysis of the native protein and its *S*-cyanoethyl derivative. In the case of α-lactalbumin the observed increase in ammonia amounted to 0.85% while the calculated increase (based on Eq. (2) and on the *S*-cyanoethylcysteine content of the protein derivative) was 0.90%. In the case of β-lactoglobulin the observed increase in ammonia was 0.50% while the calculated value was 0.48%.

DISCUSSION

Cleavage of disulfide bonds of proteins and their stabilization prior to enzymic hydrolysis for structural studies serves a double purpose: (*a*) to facilitate the separation of peptide fragments which otherwise might be held together by disulfide bonds; and (*b*) to prevent the disulfide interchange reactions (12, 13) which would render the isolation of cystine-containing peptides very uncertain.

Performic acid oxidation of disulfide bonds, which was used with success in the case of insulin (1) and ribonuclease (2), if applied to α-lactalbumin and β-lactoglobulin resulted in complete destruction of tryptophan and in very extensive damage to tyrosine and phenylalanine.

Carboxymethylation of a protein through reduction of disulfide bonds and subsequent coupling with iodoacetate, if applied to the whey proteins investigated, produced derivatives which, upon analysis, contained in addition to *S*-carboxymethylcysteine two additional carboxymethylated amino acids which were not further identified in the present work. The unspecific nature of carboxymethylation of a reduced protein with iodoacetate is not surprising in view of the recent findings of Gundlach, Stein, and Moore (14) and Barnard and Stein (15).

The two methods so far discussed for the cleavage of disulfide bonds appear to lack the specificity required in order to be useful as a general procedure.

The sulfite method proposed by Swan (4), which is based on the conversion of cystyl and cysteinyl groups into *S*-sulfocysteinyl groups, appears to be specific (16, 17) as can be judged by total sulfur determination. However, our preparation of *S*-sulfo-α-lactalbumin, which was found to be stable at mild acid and alkaline pH's, showed indication of decomposition if its enzymic digest was chromatographed using Dowex 50 × 2 resin and pH gradients between 3 and 5. The resulting effluent curve showed typical "smearing" effects which rendered the separation of the peptide fragments difficult. The possibility that the resin in combination with the acid pH employed might enhance the decomposition of the *S*-sulfocyste-

ine-containing peptides appeared to be supported by the fact that all the chromatographically isolated peptides were contaminated with cystine-containing fragments.

The method described in the present paper is based on the reduction of the disulfide bonds in proteins at pH 8.0 with β-mercaptoethanol and on the subsequent reaction with acrylonitrile. As can be concluded from Table I, this reaction resulted in the specific and complete conversion of the cysteinyl residues to *S*-cyanoethylcysteinyl groups. Upon acid hydrolysis, as used for amino acid analysis, these residues were quantitatively converted to *S*-carboxyethylcysteine and served as the basis for the analytical measurement of the extent of the reaction.

It should be noted that the complete conversion of α-lactalbumin and β-lactoglobulin to their *S*-cyanoethyl derivatives was accomplished in the absence of any denaturing agent. Whether these conditions are applicable to other proteins requires further investigation. The possible replacement of β-mercaptoethanol as a reducing agent with sodium borohydride also deserves consideration in view of the recent improvement of this method by Stark, Stein, and Moore (18) which prevents the hydrolysis of peptide bonds as a secondary reaction. The reaction involving the stabilization of reduced disulfide bonds of proteins with acrylonitrile thus was shown to be a specific one, and the inherent stable nature of the resulting *S*-cyanoethyl derivatives should render this procedure suitable for structural studies.

REFERENCES

1. SANGER, F., *Biochem. J.* **44,** 126 (1949).
2. HIRS, C. H. W., *J. Biol. Chem.* **219,** 611 (1956).
3. JOLLÈS, J., JOLLÈS, P., AND JAUREGUI-ADELL, J., *Bull. soc. chim. biol.* **42,** 1319 (1960).
4. SWAN, J. M., *Nature* **180,** 643 (1957).
5. TOMIMATSU, Y., BARTULOVICH, J. J., AND WARD, W. H., *Textile Research J.* **29,** 593 (1959).
6. GORDON, W. G., AND ZIEGLER, J., *Biochem. Preparations* **4,** 16 (1955).
7. MOORE, S., SPACKMAN, D. H., AND STEIN, W. H., *Anal. Chem.* **30,** 1185 (1958).
8. BRAND, E., SAIDEL, L. J., GOLDWATER, W. H., KASSELL, B., AND RYAN, F. J., *J. Am. Chem. Soc.* **67,** 1524 (1945).
9. GORDON, W. G., AND ZIEGLER, J., *Arch. Biochem. Biophys.* **57,** 80 (1955).
10. SPIES, J. R., AND CHAMBERS, D. C., *Anal. Chem.* **20,** 30 (1948).
11. STEIN, W. H., AND MOORE, S., *J. Biol. Chem.* **178,** 79 (1949).
12. RYLE, A. P., AND SANGER, F., *Biochem. J.* **60,** 535 (1955).
13. SPACKMAN, D. H., STEIN, W. H., AND MOORE, S., *J. Biol. Chem.* **235,** 648 (1960).
14. GUNDLACH, H. G., STEIN, W. H., AND MOORE, S., *J. Biol. Chem.* **234,** 1754 (1959).
15. BARNARD, E. A., AND STEIN, W. D., *J. Molecular Biol.* **1,** 339 (1959).
16. PECHÈRE, J-F., DIXON, G. H., MAYBURY, R. H., AND NEURATH, H., *J. Biol. Chem.* **233,** 1364 (1958).
17. WEIL, L., AND SEIBLES, T. S., *Arch. Biochem. Biophys.* **84,** 244 (1959).
18. STARK, G. R., STEIN, W. H., AND MOORE, S., *J. Biol. Chem.* **236,** 436 (1961).